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*Published in:*  
ChemBioChem

*Link to article, DOI:*  
[10.1002/cbic.201300676](https://doi.org/10.1002/cbic.201300676)

*Publication date:*  
2014

*Document Version*  
Publisher's PDF, also known as Version of record

[Link back to DTU Orbit](#)

*Citation (APA):*  
Bouhired, S. M., Crüsemann, M., Almeida, C., Weber, T., Piel, J., Schäberle, T. F., & König, G. M. (2014). Biosynthesis of Phenylannolone A, a Multidrug Resistance Reversal Agent from the Halotolerant Myxobacterium Nannocystis pusilla B150. *ChemBioChem*, 15(5), 757-765.  
<https://doi.org/10.1002/cbic.201300676>

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# Biosynthesis of Phenylannolone A, a Multidrug Resistance Reversal Agent from the Halotolerant Myxobacterium *Nannocystis pusilla* B150

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The myxobacterial strain *Nannocystis pusilla* B150 synthesizes the structurally new polyketides phenylannolone A–C. Apart from some common volatiles and siderophores, these are the first natural products from the genus *Nannocystis*. Phenylannolone A shows inhibitory activity towards the ABCB1 gene product P-glycoprotein and reverses daunorubicin resistance in cancer cells. To decipher the biochemical reactions leading to the formation of phenylannolone A, the putative biosynthetic genes were identified (phn1, phn2). Phn2 is a polyketide synthase (PKS) with an NRPS-like loading module, and its domain order is consistent with the phenylannolone A structure. The

functionality and substrate selectivity of the loading module were determined by means of a  $\gamma$ -<sup>18</sup>O<sub>4</sub>-ATP pyrophosphate exchange and a phosphopantetheine ejection assay. A specific activation of cinnamic acid by the AMP-ligase was detected. Phn1 is a putative butyryl-CoA carboxylase (BCC), providing ethylmalonyl-CoA for the formation of the ethyl-substituted part of phenylannolone A. Phn1 is the first BCC found in biosynthetic genes for an ethyl-substituted natural compound. Biosynthesis of phenylannolone A, putatively encoded by phn1 and phn2, thus utilizes the first biosynthetic machinery in which both a BCC and a PKS are involved.

## Introduction

Myxobacteria belong to the  $\delta$ -subdivision of Proteobacteria and are an important source of new biologically active metabolites, including antibacterial, antifungal, antiparasitic, and antitumor agents.<sup>[1]</sup> The most prominent examples are the epothilones, of which a semisynthetic derivative—ixabepilone (Ixemptra)—was approved in 2007 for the treatment of breast cancer.<sup>[2]</sup>

The majority of the myxobacterial compounds isolated so far are polyketides (PKs), nonribosomal peptides (NRPs), or PK/NRP hybrid molecules. These natural products are synthesized by large multifunctional enzymes: that is, polyketide synthases (PKSs) and nonribosomal peptide synthetases (NRPSs).<sup>[3,4]</sup> Out of over 30 identified myxobacterial biosynthetic gene clusters, only eight encode for pure PKSs and two for NRPSs, whereas the vast majority encode for PKS/NRPS hybrids.

Most myxobacterial PKSs and NRPSs have a modular organization, in which each module catalyzes the incorporation of one building block into the nascent molecule. Each module is further subdivided into domains: enzymatic units that are responsible for loading, condensation, and further modification of the extender unit in question. This one-to-one correspondence between domains and biosynthetic transformations is known as “colinearity”, and allows the deduction of extension cycles from the number of modules, as well as certain structural features such as the oxidation state of a building block, directly from the genetic information.<sup>[3,5,6]</sup> Conversely, a “retro-biosynthetic analysis” of a compound’s structure enables the prediction of the domains involved in its biosynthesis.

Three representatives of a new class of compounds—phenylannolones A–C (Scheme 1)—were previously isolated by our group from the halotolerant myxobacterium *Nannocystis pusilla* B150.<sup>[7]</sup> For phenylannolone A, the main metabolite of this series, inhibitory activity towards P-glycoprotein (P-gp) could be shown. P-gp is an ABC-transporter responsible for the efflux of drugs from cells, such as anticancer agents from tumor cells, which results in treatment failure. We were able to show that co-treatment of drug-resistant cancer cells with phe-


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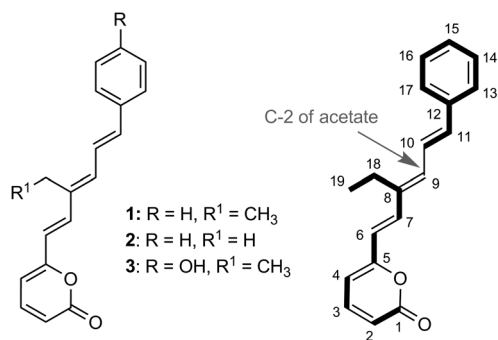
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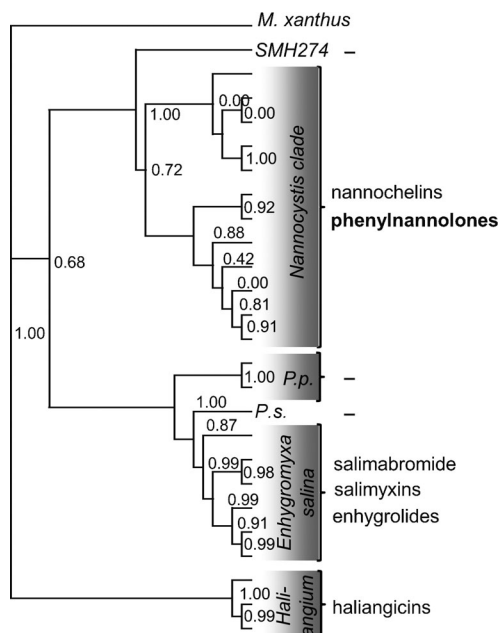
 Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/cbic.201300676>.



**Scheme 1.** Structures of phenylnannolones A (**1**), B (**2**), and C (**3**) from *N. pusilla*. Biosynthetic building blocks of phenylnannolone A (in bold). Feeding experiments in *N. pusilla* B150 with  $^{13}\text{C}$ -labeled precursors showed incorporation of a phenylalanine-derived starter unit (C10–C17), one butyrate unit (C7, C8, C18, C19), and three acetate units. The incorporation of an acetate C-2 at position 9 is unprecedented.

nylnannolone A and daunorubicin resulted in a tenfold decrease in resistance to the anticancer drug.<sup>[7]</sup> The effect of the nontoxic phenylnannolone A was thus comparable with those of P-gp inhibitors of the third generation, such as tariquidar.<sup>[8,9]</sup>

For a deeper understanding of the biochemical reactions involved in the formation of phenylannolone A, our current investigation addressed the elucidation of the biosynthetic genes for phenylannolone A. Here we present the first biosynthetic genes from myxobacteria of the genus *Nannocystis* (Figure 1).



**Figure 1.** Phylogenetic tree of halotolerant and halophilic myxobacteria, based on 16S rDNA sequences. Numbers indicate the corresponding bootstrap values. The specific natural products isolated from the corresponding clade are named on the right-hand side. The phenylannolones (**bold**) are the first compounds for which biosynthetic genes were identified. The terrestrial myxobacterium *Myxococcus xanthus* represents the outgroup. *Pp.*: *P. pacifica*. *P.s.*: *Pseudenhigromyxa salsuginis*. Complete strain names and accession numbers are given in Table S20.

## Results and Discussion

## Identification of the biosynthetic genes

Investigations into the biosynthesis of phenylannanolone A with labeled precursors revealed acetate, butyrate, and a phenylalanine-derived starter unit as building blocks (Scheme 1). Unexpectedly, acetate labeling experiments established the incorporation of the C-2 carbon atom (i.e., the methyl group) of an acetate unit in position 9.<sup>[7]</sup> From the labeling pattern it can be deduced that the lactone ring and parts of the polyene chain, including the ethyl side chain, originate from a PKS, which, due to the phenylalanine incorporation, must have an NRPS-like loading module.

Genome sequencing provided 3804 contigs with an average contig size of only 3 kb and a 16.6-fold coverage of the genome. All contigs were screened by using BLAST and CLUSEAN software for PKS genes with DNA information for ketosynthase (KS), acyltransferase (AT), dehydratase (DH), ketoreductase (KR), and thioesterase (TE) domains.<sup>[10,11]</sup> Because of the small sizes (1–2 kb) of many contigs, only PKS fragments encoding for merely one or two PKS domains could be identified. Overall, the contigs were not extensive enough to allow the assembly of even parts of a biosynthetic gene cluster.

One of the contigs, however—contig 1540, with a size of 5035 bp—included a KR domain, an acyl carrier protein (ACP), and a TE domain, and was coincident with the projected final biochemical reactions of phenylannolone A biosynthesis.

By screening of a fosmid library with primers deduced from the TE domain of contig 1540 (primer pair 1540.F/1540.R, Table S1 in the Supporting Information) the fosmid clone 12A9 was identified. This clone was then assayed by PCR (primer pair KS1up/KSd1, Table S1, Figure S1) for the presence of sequences coding for KS domains. Subsequently, the fosmid clone 12A9 was completely sequenced, thereby providing sequence information for 36839 bp.

### Sequence analysis of the phenylannolone A biosynthetic genes

Sequence analysis of fosmid 12A9 revealed 12 open reading frames (orfs) (Table 1). Two could be assigned as putatively involved in the biosynthesis of phenylannolone A and were named *phn1* and *phn2*. In a BLASTp analysis, the gene product of *phn1* showed 70% identity to the  $\beta$ -subunit of the putative propionyl-CoA carboxylase (PCC) from the myxobacterium *Plesiocystis pacifica* SIR-1 (Table 1). Such proteins act as carboxyl transferases involved in precursor supply in fatty acid or polyketide biosynthesis.<sup>[12]</sup> *Phn2* revealed 47% identity to a gene encoding for a PKS of *Streptomyces violaceusniger* Tu 4113 (Table 1). A detailed analysis of the 22 kb gene gave evidence for the presence of a complete PKS consisting of five modules. The first module contains an AMP-ligase/synthetase and an ACP domain and would therefore expected to be the loading module. The two following modules showed the domain order KS-AT-DH-KR-ACP. Extension module 3 (KS-AT-ACP) lacks the reductive domains. The last extension module, extension

**Table 1.** Deduced functions of open reading frames of the fosmid carrying the phenylannolone biosynthetic genes *phn1* and *phn2* (gray).

Gene	Size [kb]	Highest homology (protein level)	Putative function	Identity of aligned amino acids <sup>[a]</sup>	GenBank accession no.
<i>orf1</i>	1.7	hypothetical protein MYSTI_00784 [ <i>Myxococcus stipitatus</i> DSM 14675]	ANK superfamily, SMI1-KNR4 family protein	67/184 (36%)	YP_007357817.1
<i>orf2</i>	0.9	hypothetical protein [ <i>Nocardia</i> sp. BMG111209]	ANK superfamily protein	86/241 (36%)	WP_019929995.1
<i>orf3</i>	0.5	hypothetical protein SCE1572_14840 [ <i>Sorangium cellulosum</i> So0157-2]	SMI1-KNR4 family protein	87/126 (69%)	YP_008149402.1
<i>orf4</i>	0.7	hypothetical protein [ <i>Plesiocystis pacifica</i> ]	unknown	43/103 (42%)	WP_006974326.1
<i>orf5</i>	0.9	RNA polymerase [ <i>Sorangium cellulosum</i> So0157-2]	RNA polymerase	70/161 (43%)	YP_008148278.1
<i>orf6</i>	0.8	leucine-rich repeat protein [ <i>Leptospira noguchii</i> ]	unknown	71/267 (27%)	WP_004424624.1
<i>phn1</i>	1.7	propionyl-CoA carboxylase, $\beta$ -subunit [ <i>Plesiocystis pacifica</i> SIR-1]	carboxyl transferase subunit	373/535 (70%)	EDM78666.1
<i>phn2</i>	22	$\beta$ -ketoacyl synthase [ <i>Streptomyces violaceusniger</i> Tu 4113]	PKS	2174/4630 (47%)	YP_004817600.1
<i>orf7</i>	0.4	hypothetical protein [ <i>Virgibacillus</i> sp. CM-4]	unknown	28/97 (29%)	WP_021290293.1

[a] Ratio of identical amino acids (first value) to all compared amino acids (second value).

module 4, showed a domain architecture comparable to that of the extension modules 1 and 2 (KS-AT-DH-KR-ACP) plus a C-terminal TE domain, hypothesized to be responsible for the release and cyclization of the assembled molecule.

### Domains encoded on *phn2*

Multiple sequence alignment of the deduced protein sequences of the KS domains showed the presence of the highly conserved catalytic triad (C-H-H), essential for decarboxylative condensation (Figure S5), in all four cases.<sup>[13]</sup> Likewise, all five ACPs were considered active domains because they harbor the conserved serine residue (Figure S4).<sup>[14]</sup>

Each of the four extension modules of *Phn2* showed the presence of an AT domain (AT<sub>1</sub>–AT<sub>4</sub>). AT<sub>2</sub>, AT<sub>3</sub>, and AT<sub>4</sub> clearly displayed the conserved motifs of malonyl-CoA-specific AT domains (Figure S6).<sup>[15,16]</sup> AT<sub>1</sub> is, however, distinguished from the other AT domains (Figure S7) because it has the highest homology (57% identity) to an AT domain of TgaA, a PKS from *Sorangium cellulosum* (Figure S6). This TgaA AT domain, harboring a YASH as motif III, is located in the second module of the thuggacin A PKS and is reported to recognize a methylmalonate unit as its substrate.<sup>[17]</sup> Although malonyl- and ethylmalonyl-CoA-specific ATs can be distinguished reasonably reliably at the molecular level, there are fewer differences between methylmalonyl- and ethylmalonyl-CoA-specific ATs. For methylmalonyl-CoA-specific ATs, three conserved motifs (RDVVQ, GHSxG, and YASH) are described.<sup>[16]</sup> The first two motifs are nearly identical to those present in ethylmalonyl-CoA-specific ATs and thus not useful for discrimination; this leaves only the slight variations in the YASH motif. Like methylmalonyl-CoA-transferring ATs, which with a few exceptions always bear a serine as the third amino acid in the xAxH motif (motif III), those for ethylmalonyl-CoA also harbor small amino acids at

the corresponding site, but with broader variety: that is, glycine, threonine, serine, and alanine (Figure S8). The xAxH motif (motif III, Figures S6 and S8) in AT<sub>1</sub> is represented as CATH, which is a previously undescribed motif for an AT domain. The presence of threonine as the third amino acid in this motif is comparable to the situation in the ethylmalonyl-CoA-transferring ATs from oligomycin and ascomycin biosynthesis (Figure S8).<sup>[18,19]</sup> The tyrosine (Y) in the YASH motif (i.e., xAxH motif in general) is required for the formation of a hydrophobic pocket, important for binding the substrate. As AT<sub>1</sub> contains a C residue at this site, it is important to note that, in some cases, it has been reported that this first amino acid residue of the motif is altered, being replaced by, for example, a valine or a tryptophan residue.<sup>[15,16]</sup> Indeed, our multiple sequence alignments (Figure S8) revealed that in ethylmalonyl-CoA-transferring ATs valine, cysteine, threonine, or isoleucine are observed at this position. This is in accordance with AT<sub>1</sub> being an ethylmalonyl-CoA-transferring AT.

If all available data are taken together, the xAxH motif—that is, CATH in AT<sub>1</sub>—appears to have a preference for ethylmalonyl-CoA as substrate. This hypothesis is supported by our feeding experiments with 1-<sup>13</sup>C-butyrate, which was incorporated into phenylannolone A to form the ethyl side chain of the metabolite.<sup>[7]</sup> Furthermore, substrate preference for ethylmalonyl-CoA is corroborated by the production rates of the different phenylannolone derivatives. Observed production of the ethyl-branched phenylannolone A was seven times higher than that for the methyl-branched phenylannolone B.<sup>[7]</sup> The occurrence of phenylannolone B suggests that AT<sub>1</sub> can also activate methylmalonyl-CoA, but only to a minor extent. This activity might result not only from a higher specificity of AT<sub>1</sub> towards ethylmalonyl-CoA, but possibly also from a better supply of the precursor ethylmalonyl-CoA putatively provided by *Phn1*.

Sequence analysis of the three ketoreductase (KR) domains in Phn2 revealed the presence of the Rossmann fold motif GxGxxG and the K-S-Y catalytic triad, required for activity (Figures S9 and S10). The highly conserved active site motif Dxxx(Q/H), responsible for catalyzing the elimination of the  $\beta$ -hydroxy group to generate an  $\alpha,\beta$ -unsaturated moiety, was identified for all dehydratase (DH) domains of Phn2 (Figure S11). For the motif LxxHxxxGxxxxP, however, mutations of the conserved leucine (in the DH<sub>2</sub> domain) and glycine (in DH<sub>1</sub> and DH<sub>2</sub>) were observed (Figure S11).<sup>[20]</sup>

The KR domains of the phenylannolone A biosynthetic genes were characterized by analyzing several conserved motifs that allowed stereochemical prediction.<sup>[21,22]</sup> KR<sub>1</sub> and KR<sub>2</sub> were identified as B1-type KR domains, resulting in a  $\alpha$ -hydroxy-substituted thioester and, through the action of the neighboring DH domain, finally in a *trans* double bond. KR<sub>4</sub>, however, lacks the LDD motif (motif II) present in KR<sub>1</sub> and KR<sub>2</sub> that is found in "B-type" KRs, but also lacks the conserved tryptophan residue (motif III) present in "A-type" KRs (Figure S9). Although tryptophan is present in nearly all A-type KRs, there are a few examples of KR domains that generate  $\alpha$ -hydroxy groups despite a missing tryptophan motif (i.e., the corresponding domains in stigmatellin, chivosazol, and disorazole biosynthesis).<sup>[23–25]</sup> Furthermore, KR<sub>4</sub> from Phn2 showed (in a BLAST analysis) the highest identity to a KR from StiD, involved in stigmatellin biosynthesis in *Stigmatella aurantiaca*. This KR from StiD converts the  $\beta$ -keto group to an  $\alpha$ -hydroxy group, which in this case is subsequently methylated through the action of a methyltransferase (Figures S9 and S17).<sup>[23]</sup> Our conclusions are additionally supported by the fact that in chivosazol biosynthesis KR<sub>4</sub> and KR<sub>14</sub> are classified as A-type KRs, which catalyze the formation of *cis* double bonds. Both KRs lack the conserved tryptophan residue, which is replaced by leucine and valine, respectively.<sup>[24]</sup> A third example of a set of A-type KRs lacking the tryptophan motif is found in the disorazole (Figure S17) gene cluster, in which the tryptophan residues in KR<sub>4</sub>, KR<sub>5</sub>, and KR<sub>7</sub> are replaced by valine, methionine, and leucine, respectively.<sup>[25]</sup> If we take note of all these examples of A-type KRs without the typical tryptophan motif, it is very likely that KR<sub>4</sub> in Phn2 generates an  $\alpha$ -hydroxy group, and that this is further processed by DH<sub>4</sub> to afford a *cis* double bond. For the biosynthesis of phenylannolone A a *cis* configuration in this position is necessary to facilitate the formation of the lactone ring.

The TE domain located at the C terminus of Phn2 included the conserved motif GxSxG and the active site triad S-D-H (Figure S12).<sup>[26]</sup> For the formation of the terminal pyrone ring of phenylannolone A, keto–enol tautomerism of the unreduced carbonyl group (module 3), resulting in an enol functionality, would be expected (Figure S18). TE-induced pyrone ring formation is uncommon for modular type I PKSs. Triketide lactonization has previously been studied in the case of DEBS 1-TE, a truncated form of the erythromycin polyketide synthase.<sup>[27,28]</sup> A further example of pyrone formation in a type I PKS, following a mechanism similar to that proposed for phenylannolone A, is represented in jerangolid biosynthesis.<sup>[29]</sup>

## Phn1 and precursor supply with ethylmalonyl-CoA

PKS extender units such as methoxymalonyl-CoA, hydroxymalonyl-CoA, and ethylmalonyl-CoA are less common in polyketide biosynthesis, and their supply is a limiting factor in such biosynthetic processes.<sup>[30]</sup>

Ethylmalonyl-CoA can be produced from crotonyl-CoA through the action of the enzyme crotonyl-CoA carboxylase/reductase (CCR), belonging to the ethylmalonyl-CoA pathway.<sup>[31,32]</sup> Because only a few bacteria have an operating ethylmalonyl-CoA pathway (apart from secondary metabolic pathways), most biosynthetic gene clusters for ethyl-substituted polyketides harbor a gene encoding a CCR homologue: such as, concanamycin, oligomycin, or tylosin.<sup>[18,33,34]</sup>

Other proposed pathways for the formation of ethylmalonyl-CoA require the carboxylation of butyryl-CoA. Butyryl-CoA might be derived either from L-valine, which is catabolized to isobutyryl-CoA and then converted into butyryl-CoA, or from  $\beta$ -oxidation of even-chain fatty acids.<sup>[35,36]</sup> In each case acyl-CoA carboxylases are responsible for the carboxylation reaction (Figure S16).

The biosynthetic gene clusters of indanomycin and tiacumicin were found to harbor both types of enzymes: that is, the CCR and the acyl-CoA carboxylase. In the case of indanomycin the involvement of an acyl-CoA carboxylase (IdmB) responsible for the supply of both ethylmalonyl-CoA and methylmalonyl-CoA was discussed. This enzyme harbors an isoleucine at position 422, the active site residue. It was thus postulated that IdmB accepted substrates such as acetyl-CoA, propionyl-CoA, and butyryl-CoA with almost equal affinities, just like acetyl-CoA carboxylases.<sup>[37,38]</sup> For the precursor supply in tiacumicin B biosynthesis, three genes encoding for enzymes of the ethylmalonyl-CoA pathway—a hydroxybutyryl-CoA dehydrogenase (TiaJ), a crotonyl-CoA hydratase (TiaN), and a crotonyl-CoA carboxylase (TiaK)—were identified. In addition to these three enzymes that form ethylmalonyl-CoA, another enzyme, a PCC encoded by *tiaL*, is believed to supply the PKS with methylmalonyl-CoA.<sup>[39]</sup> Multiple sequence alignment with sequences of different PCCs revealed the active site residue for TiaL to be an isoleucine (Figure S2). It was thus suggested that the affinity of this PCC was comparable with that of IdmB. This conclusion is supported by crystal structure and mutational analysis of the  $\beta$ -subunit of a PCC from *Streptomyces coelicolor*.<sup>[37,40]</sup>

With regard to the biosynthesis of phenylannolone A, the supply of ethylmalonyl-CoA putatively results from the enzymatic reaction of Phn1, a proposed  $\beta$ -subunit of a carboxylase (Table S2). This  $\beta$ -subunit acts as a carboxyl transferase, whereas the  $\alpha$ -subunit harbors the biotin-carboxylase (BC) and biotin-carboxylase-carrier protein (BCCP) domain.<sup>[37,41]</sup> IdmB from *Streptomyces antibioticus* also displays only the  $\beta$ -subunit of an acyl-CoA carboxylase. Li et al. discussed the formation of an active complex with partnering BC and BCCP, encoded elsewhere within the genome of the strain in question.<sup>[38]</sup>

The active site residue of Phn1 is an alanine unit, rather than the conserved aspartate residue found in PCCs (Figure S2). Mutational experiments for a PCC in *S. coelicolor* showed that mutations of this type led to a clear shift in substrate preference

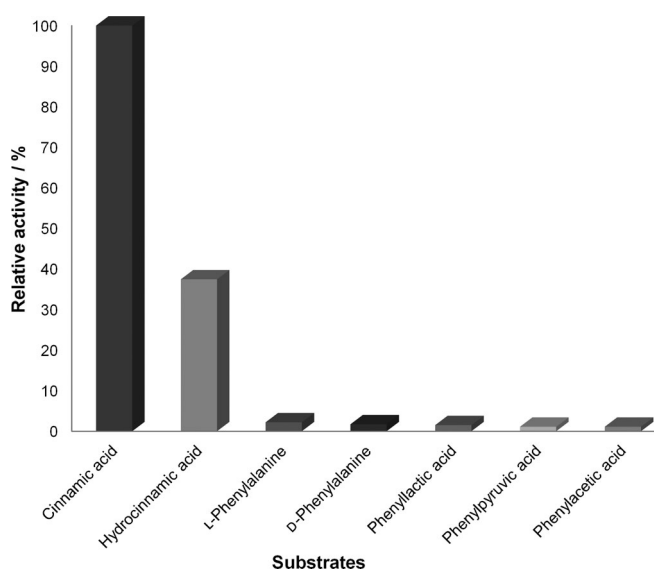


from propionyl-CoA to butyryl-CoA.<sup>[37,40]</sup> Phn1 is thus more likely a butyryl-CoA carboxylase (BCC) than a PCC, and hence preferentially provides ethylmalonyl-CoA for the PKS assembly line. This is also corroborated by the ratio of amounts of phenylannolone A (2.8 mg L<sup>-1</sup>) and phenylannolone B (0.4 mg L<sup>-1</sup>) produced *in vivo*, showing a sevenfold higher prevalence for ethylmalonyl-CoA over methylmalonyl-CoA.<sup>[7]</sup>

Phn1 is to the best of our knowledge the first putative BCC found in a biosynthetic gene cluster for an ethyl-substituted natural compound.

### Biochemical characterization of the AMP-ACP didomain

The functionality of the biosynthetic genes was tested by biochemical characterization of distinct enzymes because *N. pusilla* B150 is not yet genetically accessible and thus no standard protocols for mutagenesis are available for this species. A putative loading module forms the N-terminal part of Phn2, it consists of a didomain: that is, an AMP-ligase (Figure S3, Table S2) and an ACP. To investigate the substrate selectivity of the putative AMP-ligase, heterologous expression of the recombinant apo-AMP-ACP didomain was projected with the goal of performing ATP-PP<sub>i</sub> exchange assays. The assay, described by Phelan et al., measures the isotopic back exchange of unlabeled pyrophosphate into  $\gamma$ -<sup>18</sup>O<sub>4</sub>-labeled ATP.<sup>[42]</sup> To obtain the 80 kDa apo-AMP-ACP didomain, pet151AMPACP was expressed in *E. coli* BL21, and expression was confirmed by SDS-PAGE (Figures S13 and S14). We expected from the isotope labeling studies that either a C<sub>6</sub>C<sub>2</sub> or a C<sub>6</sub>C<sub>3</sub> moiety forms the starter unit, and so phenylacetic acid (C<sub>6</sub>C<sub>2</sub>) and several phenylpropanoids (C<sub>6</sub>C<sub>3</sub>) were tested in the ATP-PP<sub>i</sub> exchange assay. For the apo-AMP-ACP didomain, selectivity towards cinnamic acid was observed, with an ATP-PP<sub>i</sub> exchange of 47% being measured for this C<sub>6</sub>C<sub>3</sub> unit (Figure 2). Hydrocinnamic acid showed only a third of this exchange activity (17.7%). All other applied substrates—phenylacetic acid, D-/L-phenylalanine, phenyllactic acid, and phenylpyruvic acid—did not reach more than 1.1% exchange activity (Figure S19, Table S4). Characterization of the loading didomain (A-PCP) from McyG (Table S3), an NRPS/PKS system involved in microcystin biosynthesis, was reported to show divergent results when the ATP-PP<sub>i</sub> exchange assay with apoenzyme or alternatively a mass spectrometric analysis with holoenzyme was employed.<sup>[43]</sup> To confirm the results of our ATP-PP<sub>i</sub> exchange assay and to characterize the substrate loaded onto the phosphopantetheine (PPant) arm of the loading didomain (AMP-ACP) directly, loading studies were performed on the holoenzyme. The phosphopantetheinylated holo-AMP-ACP was obtained by expression of pet151AMPACP in *E. coli* Bap-1 cells. This protein was used in a PPant ejection assay with different substrates.<sup>[44]</sup> In addition to phenylacetic acid (C<sub>6</sub>C<sub>2</sub>), several phenylpropanoids (C<sub>6</sub>C<sub>3</sub>), such as cinnamic acid, hydrocinnamic acid, *p*-coumaric acid, D-/L-phenylalanine, phenyllactic acid, and phenylpyruvic acid, were tested. Mass spectrometric analysis showed that the loading of three substrates was successful: that is, cinnamic acid, *p*-coumaric acid, and phenyllactic acid (Scheme 2). On provision of a mixture of cinnamic acid and phenyllactic acid to the holoenzyme, only



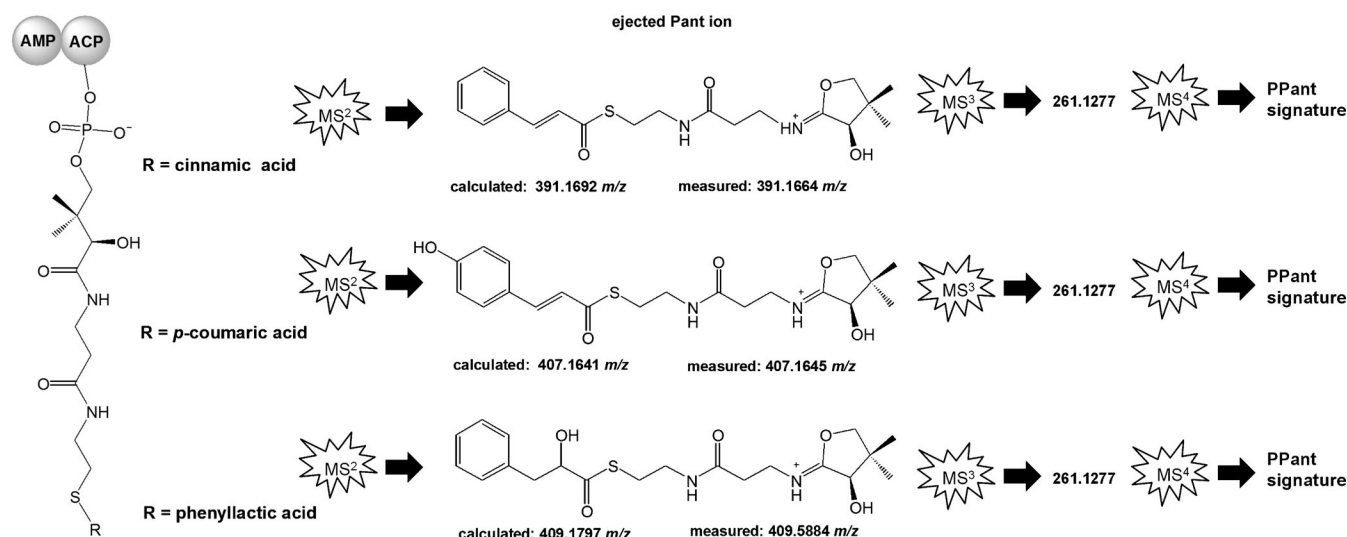
**Figure 2.** Relative activities of the AMP domain from Phn2, measured in an  $\gamma$ -<sup>18</sup>O<sub>4</sub>-ATP pyrophosphate exchange assay.

cinnamic acid was loaded to the PPant arm of the AMP-ACP didomain.

From the results of these assays we can conclude that, for the formation of phenylannolone A, cinnamic acid is adenylated by the AMP-ligase and subsequently loaded to the neighboring ACP domain. The activation and loading of *p*-coumaric acid by the holo-AMP-ACP didomain explains the formation of phenylannolone C, which possesses an additional hydroxy group on the phenyl moiety and is only produced in trace amounts by *N. pusilla* B150.

With regard to our previous feeding experiments, which suggested the incorporation of a phenylacetic acid unit rather than cinnamic acid, there are still some open questions to be answered. In this context, it is useful to refer to similar experiments with the cyanobacterium *Microcystis aeruginosa*, in which phenylalanine was incorporated into microcystin in the same manner.<sup>[43]</sup> The same applies to the starter units in the biosynthesis of nodularin and cryptophycin, both isolated from cyanobacteria.<sup>[45–47]</sup> Biochemical characterization of the A-PCP didomain from McyG, an NRPS/PKS system involved in microcystin biosynthesis, revealed that several phenylpropanoids (C<sub>6</sub>C<sub>3</sub>) are preferentially activated and loaded onto the PCP domain, rather than the postulated phenylacetic acid (C<sub>6</sub>C<sub>2</sub>). Indeed, *in vivo* loading experiments pointed towards 3-phenyllactate, now considered to be the true starter unit of microcystin.<sup>[43]</sup> However, a remaining mystery is the mechanistic basis for the one-carbon truncations from C<sub>6</sub>C<sub>3</sub> to C<sub>6</sub>C<sub>2</sub> units in the biosyntheses of microcystin, cryptophycin, nodularin, and, in our case, phenylannolone.<sup>[43,47]</sup>

The biosynthesis of cinnamic acid in a few bacteria—that is, *Streptomyces maritimus*, *Photobacterium luminescens*, *Legionella* spp., and two strains of cyanobacteria (*Anabaena variabilis* and *Nostoc punctiforme*)—is mediated by a phenylalanine ammonia lyase (PAL).<sup>[48–51]</sup> In a single reaction, PAL transforms phenylalanine into *trans*-cinnamic acid, with every carbon atom being preserved and no carbon cleavage taking place. However, this



**Scheme 2.** Pantetheine (Pant) ejection assay on the holo-AMP-ACP loading didomain. Acylation of the holo-AMP-ACP didomain was observed for three substrates: cinnamic acid, *p*-coumaric acid, and phenyllactic acid, with ejection ions of  $m/z$  391.1664,  $m/z$  407.1645, and  $m/z$  409.5884 being measured by FTMS. Correspondingly, MS<sup>3</sup> resulted in signature ions of the Pant arm with the mass of  $m/z$  261.1277. MS<sup>4</sup> of the substrate fragment yielded another series of corresponding signature ions ( $m/z$  243.2 to 142.1; see Meluzzi et al., 2008)<sup>[44]</sup> verifying the presence of the PPant arm. Substrates such as phenylacetic acid, phenylpyruvic acid, L-phenylalanine, D-phenylalanine, and hydrocinnamic acid were not loaded.

does not explain the unusual results of our feeding experiments, in which a single carbon atom, derived from acetate, is incorporated. The formation of the starter unit (that is, cinnamic acid) in *N. pusilla* B150 remains elusive and probably includes a new pathway, a suggestion for which is presented in Figure S15. Cinnamoyl-CoA is quite a common starter molecule in type III PKSs of plants and bacteria, as observed for stilbene, curcumin, and flavonoid biosynthesis.<sup>[52,53]</sup> For the modular type I PKSs however, cinnamoyl-CoA is an uncommon starter unit, although recently reported to be involved in the PKS assembly line of legiolin.<sup>[51]</sup>

### Model for phenylannolone A biosynthesis

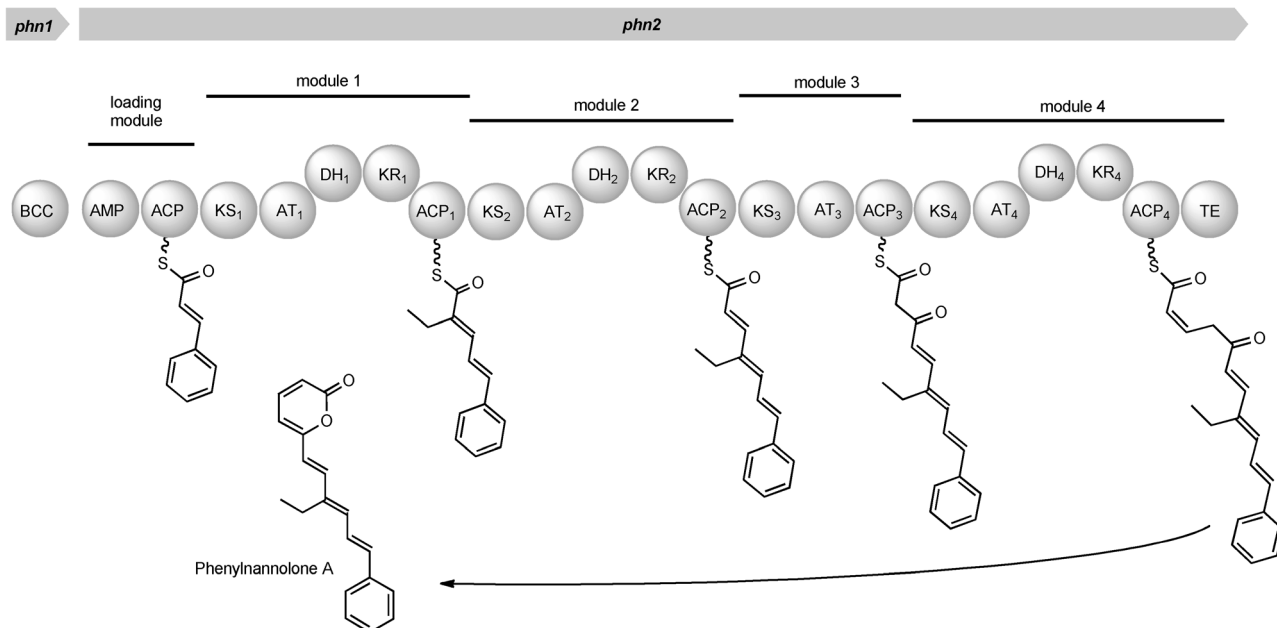
The putative biosynthetic genes for phenylannolone A consist of a putative carboxyl transferase gene (*phn1*) and a modular type I PKS gene (*phn2*).

It is proposed that the carboxyl transferase, encoded by *phn1*, is responsible for the supply of the ethylmalonyl-CoA precursor. The adjacent PKS enzyme consists of five modules: a loading module and four extension modules. From the domain order of the PKS encoded by *phn2* a colinear biosynthetic pathway for the formation of phenylannolone A can be proposed (Scheme 3). The loading module, comprising an AMP-dependent ligase/synthetase (AMP), thus adenylates the putative starter cinnamic acid and hence enables its loading to the adjacent acyl carrier protein (ACP<sub>LM</sub>) domain. This was shown experimentally in an ATP-PP<sub>i</sub> exchange assay and through MS-based ACP loading studies. In the following step, KS<sub>1</sub> of the first extension module prolongs the starter unit with a butyrate moiety, loaded by AT<sub>1</sub>, in a decarboxylative Claisen condensation, and this is followed by reduction and dehydration, through the action of the corresponding KR<sub>1</sub> and DH<sub>1</sub> domains, to form a *trans*-configured C=C double bond. In the

second elongation step (module 2), the growing chain is extended with an acetate unit, and this is then reduced and dehydrated in the same manner as in the step before. The third elongation module is also responsible for the extension of the polyketide chain with an acetate unit, with the difference that the  $\beta$ -keto group is not further reduced. This feature is apparent from the domain order, because no reductive domains are present in this module. The extender unit of the last step is again malonyl-CoA, which elongates the polyketide chain with a final acetate unit. The corresponding KR reduces the  $\beta$ -keto group to an L-hydroxy group, and this is then eliminated through the action of DH<sub>4</sub> to form a *cis* double bond. The stereochemistry generated in this way is necessary for the lactone ring formation and thereby for the release of the polyketide from the assembly line through the thioesterase domain.

### Conclusions

We have presented the first biosynthetic pathway for a natural compound from the genus *Nannocystis*. For the biosynthesis of the structurally novel and bioactive phenylannolones, we conclude that phenylannolone A is the product of an unusual PKS system in *N. pusilla* B150. Bioinformatic analysis of *phn1* and *phn2* showed that the domain order of the identified genes is consistent with the chemical structure of phenylannolone A and also explains the occurrence of minor metabolites. In addition, biochemical studies on the loading module of the putative phenylannolone gene product Phn2 were performed, and indicate cinnamic acid to be the starter unit. To the best of our knowledge this represents the first example of a BCC/PKS system in microbial biosynthesis.



**Scheme 3.** Proposed pathway for the biosynthesis of phenylinnanolone A. BCC: butyryl-CoA carboxylase. AMP: AMP-dependent ligase. ACP: acyl carrier protein. KS: ketosynthase domain. AT: acyltransferase domain. DH: dehydratase domain. KR: ketoreductase domain. TE: thioesterase domain.

## Experimental Section

**Biological material:** The myxobacterial strain B150 was isolated by Birgit Ohlendorf from a soil sample collected from the intertidal region of Crete, Greece. The 16S rDNA sequence of the myxobacterial strain was closely related to that of *N. pusilla* strain DSM 14622T (GenBank accession: FR749907.1) with an identity between the two sequences of 98%. This outcome supports the previous classification of this strain to the genus of *Nannocystis*.<sup>[7]</sup> Whereas Ohlendorf et al., however, suggested the species to be *Nannocystis exedens*, our result points towards *N. pusilla* B150. For bacterial growth, a cryo-culture (250  $\mu$ L) of *N. pusilla* B150 was placed on VY/2 agar plates. For cultivation in liquid medium, slices of agar, containing fruiting bodies of the myxobacterium, were inoculated in MD-1 glucose medium and grown at 30 °C for seven days under shaking conditions (140 rpm). For long-term storage at –80 °C, the liquid bacterial cultures were mixed with an equal volume of casitone (1 %).

**General procedure:** *N. pusilla* strain B150 (Figure S23 for 16S rDNA) was grown in liquid MD1+glucose medium at 30 °C (140 rpm) for seven days. *E. coli* XL1Blue was used as a host for routine cloning and construction of targeting plasmids. *E. coli* strains were grown in lysogeny broth (LB) supplemented with ampicillin at a final concentration of 100  $\mu$ g mL<sup>–1</sup>. All restriction enzymes and T4 DNA ligase were purchased from MBI Fermentas. DNA polymerases and dNTPs for PCR were obtained from Promega. Oligonucleotides were purchased from MWG-Biotech AG (Ebersberg, Germany). Gel electrophoresis materials were supplied from Neolab (Heidelberg, Germany). All other materials and substances were supplied by Roth. Isolation of plasmid DNA and chromosomal DNA, as well as *E. coli* strain transformation, were performed by standard methods.<sup>[54]</sup> DNA fragments were isolated from agarose gels with a QIAquick Gel Extraction Kit (Qiagen). For preparation of DNA templates for sequencing, the pGEMT vector (Promega) was used. Plasmid DNA purification was performed with a GeneJET Plasmid Miniprep Kit (Fermentas) or the PureYield Miniprep System (Promega). PCR was carried out with Taq DNA poly-

merase or Pfu-DNA polymerase (Promega). DMSO was added to the reaction mixture to a final concentration of 5%. Conditions for amplification with use of a Thermocycler TGradient (Biometra, Göttingen, Germany) were as follows: initial denaturation for 120 s at 95 °C, annealing for 30–45 s at 45–60 °C, extension for 60–90 s at 72 °C, denaturation for 30 s at 95 °C, 30 cycles, and a final extension for 5 min at 72 °C. PCR products were purified with a QIAquick PCR Purification Kit (Qiagen).

**Construction of the fosmid library:** Chromosomal DNA derived from a 500 mL culture of *N. pusilla* B150 was used for the construction of a fosmid library with use of pCC1FOS (Epicentre, Madison, USA). The genomic library was constructed according to manufacturer's instructions, and 2300 colonies with an average insert length of 36 kb were generated and transferred to 95-well microtiter plates. For long-term storage at –80 °C the fosmid cultures were mixed with an equal volume of glycerol (100%).

**Screening of the fosmid library:** The fosmid gene library was screened by whole-cell PCR for the presence of PKS-TE domain positive clones with use of the specific primer pair 1540.F/1540.R deduced from sequence data of contig 1540 (obtained from 454 genome sequencing steps). In order to make the fosmid DNA accessible for the Taq-polymerase, a heating step was integrated into the previously described standard PCR program. This denaturation step at the beginning of the PCR procedure was performed at 95 °C for 20 min to initiate cell lysis (whole-cell PCR). To facilitate fast screening of the library, all 96 clones of each microtiter plate were combined in 0.5 mL Eppendorf tubes. Whole-cell PCRs were set and performed with 1  $\mu$ L of each pool. PCR probes were subsequently analyzed by agarose gel electrophoresis for a fragment of 800 bp. Positive clones were then analyzed in a second PCR with the deduced KS primer pair for existing ketosynthase domains, necessary for the PKS assembly line. Degenerate PKS primers were deduced from conserved sequence motifs of KS domains, obtained from 454 genome sequencing steps, yielding fragments of 700 bp in the PCR.



**Sequencing of genomic DNA and fosmid clone of *N. pusilla* B150:** Genome sequencing was carried out by GATC Biotech AG (Konstanz, Germany) with a Roche Genome Sequencer FLX Titanium (F. Hoffmann–La Roche). Raw sequences were assembled by the company; this resulted in a draft genome with 3804 contigs of an average contig size of 3 kb. Complete sequencing of fosmid 12A9 was performed by IIT-BiotechGmbH (Bielefeld, Germany). For this purpose a high-throughput library with an insert length of 500 bp was constructed and subsequently analyzed in a pyrosequencing reaction with a Roche GS FLX Titanium+ instrument. Samples were sequenced with a Roche 454 GS FLX Titanium Sequencer (Roche). The sequence of the phenylannolone genes were deposited in Genbank with accession number KF739396.

**Bioinformatic analysis of sequences:** The software programs Clone Manager 9, CLUSEAN, and Artemis were used to attend DNA sequences. The BLAST program provided by NCBI was used for sequence data analysis. The multiple alignments of nucleotide sequences were generated with the program ClustalW powered by EMBL. For the construction of the phylogenetic tree in Figure 1, sequences were aligned by using the heuristic Smith–Waterman algorithm with mafft. Subsequently, the multiple sequence alignment was filtered with Gblocks for conserved regions. The phylogenetic tree was constructed by use of FastTree. FastTree computes local support values with the Shimodaira–Hasegawa test ( $\approx$  bootstrap values). For the construction of the phylogenetic trees in the Supporting Information the online tool provided by Phylogeny.fr was used.<sup>[55,56]</sup>

**Expression and purification of the loading didomain:** The DNA sequence of the loading didomain was amplified from the DNA of the fosmid clone 12A9 by PCR by using the primer pair revAMP-ACP/forAMPACP (Eurofins MWG Operon) and a pfu-Polymerase (Promega). The resulting PCR fragment (AMP-ACP) of 2.26 kb encoding for the two domains was cloned into the pet151/D-TOPO cloning vector (Invitrogen) and transferred into One Shot TOP10 Chemically Competent *E. coli* cells (Invitrogen). To exclude the possibility of mutations in the reading frame, the chosen clones were submitted for sequencing. Plasmid DNA (pet151AMPACP) was isolated from confirmed clones and transformed into BL21 Star(DE3) One Shot Chemically Competent *E. coli* cells (Invitrogen). For a pre-culture, LB (10 mL) with ampicillin ( $100 \mu\text{L mL}^{-1}$ ) was inoculated with transformed cells ( $500 \mu\text{L}$ ), and these were grown overnight at  $37^\circ\text{C}$ . For the main culture, LB (100 mL) containing ampicillin ( $100 \mu\text{L mL}^{-1}$ ) was inoculated with the overnight culture (2 mL). After inoculation, the main culture was grown to an  $\text{OD}_{600}$  value of  $\approx 0.5$ . Protein expression was induced by adding isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. The expression of the recombinant protein took place at  $37^\circ\text{C}$  and was stopped after 4 h. Cells were harvested and resuspended in lysis buffer [ $\text{NaH}_2\text{PO}_4$  (50 mM), NaCl (300 mM), imidazole (10 mM), pH 8.0]. Cells were lysed with the help of a Branson Sonifier 250, set to output level 4, 50% duty cycle. The samples were sonified four to five times with ten pulses each. Between the pulses the cells were placed back on ice to avoid overheating of the sample. Cell debris and insoluble parts were pelleted by centrifugation for 10 min at  $10016g$  and  $4^\circ\text{C}$ . The supernatant containing the soluble proteins and the pellet were collected. The supernatant containing the His-tagged protein was bound to Ni-NTA spin columns (Qiagen), and the pure AMP-ACP didomain was eluted with elution buffer [ $\text{NaH}_2\text{PO}_4$  (50 mM), NaCl (300 mM), imidazole (150 mM), pH 8.0], according to the manufacturer's instructions. Protein was used directly in assays. For the expression of the holo-AMP-ACP, pet151AMPACP was transferred into chemically competent BAP-

1 *E. coli* cells. The cells were cultured and purified under the same conditions as the apo-AMP-ACP protein. All proteins were analyzed by separation through a 12% SDS-PAGE gel [Tris-HCl (pH 6.8, 1 M, 2.5 mL), SDS (10%, 0.1 mL), bis-acrylamide (30%, 4 mL),  $\text{H}_2\text{O}$  (3.3 mL), APS (10%, 0.1 mL), TEMED (4  $\mu\text{L}$ )] and visualized by Coomassie Blue staining.

**Adenylation enzyme characterization by  $\gamma$ - $^{18}\text{O}_4$ -ATP pyrophosphate exchange:** In this assay the isotopic back exchange of unlabeled pyrophosphate into  $\gamma$ - $^{18}\text{O}_4$ -labeled ATP was measured.<sup>[42]</sup> Reactions were each performed in a total volume of 6  $\mu\text{L}$  [ $\text{MgCl}_2$  (5 mM),  $\text{PP}_i$  (5 mM),  $\gamma$ - $^{18}\text{O}_4$ -ATP (1 mM, or  $^{16}\text{O}_4$ -ATP as a positive control), Tris-HCl (pH 7.5, 20 mM), and substrate (1 mM)]. The reactions were carried out for 2 h at room temperature after addition of the purified A domain, and were then stopped by the addition of the same volume of 9-aminoacridine in acetone (10  $\text{mg mL}^{-1}$ ). As a negative control, instead of enzyme, buffer was added to the reaction mixture. Samples were then analyzed by MALDI-TOF MS.

**Adenylation enzyme characterization by mass-based PPant ejection assay:** A typical sample (50  $\mu\text{L}$  total volume) consisted of ATP (3 mM), A domain buffer [Tris-HCl (pH 7.5, 20 mM), glycerol (5%)], substrate (10 mM), and holo-AMP-ACP (15  $\mu\text{L}$ ), and was incubated for 30 min at room temperature. The assay mixture was placed on ice, before being subjected to semipreparative RP-HPLC (C4 reversed-phase column, Symmetry 300, 5  $\mu\text{m}$ , 4.6  $\text{mm} \times 250 \text{ mm}$ , Waters, eluent water/acetonitrile (70:30) to water/acetonitrile (35:65) over 35 min, flow rate  $1.0 \text{ mL min}^{-1}$ ). The retention time of the protein loaded with substrate was 29 min. The protein-containing fraction was collected manually and dried in a SpeedVac concentrator.

**MS<sup>n</sup> analysis:** The lyophilized samples were dissolved in a suitable volume of electro spray solution [ $\text{H}_2\text{O}$  (49.5%), methanol (49.5%), formic acid (1%)]. Use of a Thermo LTQ Orbitrap Velo coupled with an Advion TriVersa NanoMate enabled a continuous electron spray for the MS<sup>n</sup> experiments. Samples (20–50  $\mu\text{L}$ ) were loaded onto the 96-well plate of the NanoMate spray robot coupled to the LTQ Orbitrap Velos. A spray chip (5  $\mu\text{m}$  nozzle diameter) was used at a spray voltage of 1.6 kV and 0.3 psi pressure setting. An environmental polysiloxane ion with  $m/z$  445.12003 was used as lock mass for internal calibration. Isolation and fragmentation were performed in the linear ion trap, and detection of the final product spectrum was achieved with the Orbitrap analyzer. For holo-AMP-ACP with a mass of 80 kDa the ejection ions were obtained by in-source fragmentation, with application of 50–65 V fragmentation energy. The resulting ejection ions were isolated and subjected to a further round of fragmentation ( $\text{MS}^3$ ) to liberate the PPant arm (calculated  $m/z$  261.1267). To verify that these ions (measured  $m/z$  261.1277) really represent the PPant arm, they were subjected to an additional round of fragmentation ( $\text{MS}^4$ ), and the ions obtained were analyzed for the typical PPant signature.<sup>[20]</sup>

## Acknowledgements

Research was funded by the NRW International Research Graduate School Biotech-Pharma, the German Centre for Infection Research (DZIF) and the DFG Research Unit FOR 854.

**Keywords:** biosynthesis • myxobacteria • *Nannocystis pusilla* • phenylannolone A • polyketides

[1] M. Nett, G. M. König, *Nat. Prod. Rep.* **2007**, *24*, 1245–1261.

- [2] A. Conlin, M. Fournier, C. Hudis, S. Kar, P. Kirkpatrick, *Nat. Rev. Drug Discovery* **2007**, *6*, 953–954.
- [3] J. Staunton, K. J. Weissman, *Nat. Prod. Rep.* **2001**, *18*, 380–416.
- [4] S. C. Wenzel, R. Müller, *Nat. Prod. Rep.* **2009**, *26*, 1385–1407.
- [5] K. Blin, M. H. Medema, D. Kazempour, M. A. Fischbach, R. Breitling, E. Takano, T. Weber, *Nucleic Acids Res.* **2013**, *41*, W204–W212.
- [6] J. Piel, *Nat. Prod. Rep.* **2010**, *27*, 996–1047.
- [7] B. Ohlendorf, S. Leyers, A. Krick, S. Kehraus, M. Wiese, G. M. König, *ChemBioChem* **2008**, *9*, 2997–3003.
- [8] E. Fox, S. E. Bates, *Expert Rev. Anticancer Ther.* **2007**, *7*, 447–459.
- [9] B. Ohlendorf, *PhD thesis*, University of Bonn (Germany), <http://www.hut-verlag.de/9783899638639.html>, **2008**.
- [10] T. Weber, C. Rausch, P. Lopez, I. Hoof, V. Gaykova, D. H. Huson, W. Wohlleben, *J. Biotechnol.* **2009**, *140*, 13–17.
- [11] “antismash/clusean” can be found under <https://bitbucket.org/antismash/clusean>, **2012**.
- [12] A. V. Demirev, A. Khanal, N. P. K. Hanh, K. T. Nam, D. H. Nam, *J. Microbiol.* **2011**, *49*, 407–412.
- [13] Y.-M. Zhang, J. Hurlbert, S. W. White, C. O. Rock, *J. Biol. Chem.* **2006**, *281*, 17390–17399.
- [14] S. C. Findlow, C. Winsor, T. J. Simpson, J. Crosby, M. P. Crump, *Biochemistry* **2003**, *42*, 8423–8433.
- [15] G. Yadav, R. S. Gokhale, D. Mohanty, *J. Mol. Biol.* **2003**, *328*, 335–363.
- [16] S. Smith, S.-C. Tsai, *Nat. Prod. Rep.* **2007**, *24*, 1041–1072.
- [17] K. Buntin, H. Irschik, K. J. Weissman, E. Luxemburger, H. Blöcker, R. Müller, *Chem. Biol.* **2010**, *17*, 342–356.
- [18] S. Omura, H. Ikeda, J. Ishikawa, A. Hanamoto, C. Takahashi, M. Shinose, Y. Takahashi, H. Horikawa, H. Nakazawa, T. Osonoe, H. Kikuchi, T. Shiba, Y. Sakaki, M. Hattori, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 12215–12220.
- [19] K. Wu, L. Chung, W. P. Revill, L. Katz, C. D. Reeves, *Gene* **2000**, *251*, 81–90.
- [20] S.-C. Tsai, B. D. Ames, *Methods Enzymol.* **2009**, *459*, 17–47.
- [21] A. T. Keatinge-Clay, *Chem. Biol.* **2007**, *14*, 898–908.
- [22] P. Caffrey, *ChemBioChem* **2003**, *4*, 654–657.
- [23] N. Gaitatzis, B. Silakowski, B. Kunze, G. Nordsiek, H. Blöcker, G. Höfle, R. Müller, *J. Biol. Chem.* **2002**, *277*, 13082–13090.
- [24] O. Perlova, K. Gerth, O. Kaiser, A. Hans, R. Müller, *J. Biotechnol.* **2006**, *121*, 174–191.
- [25] R. Carvalho, R. Reid, N. Viswanathan, H. Gramajo, B. Julien, *Gene* **2005**, *359*, 91–98.
- [26] S.-C. Tsai, L. J. W. Miercke, J. Krucinski, R. Gokhale, J. C.-H. Chen, P. G. Foster, D. E. Cane, C. Khosla, R. M. Stroud, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 14808–14813.
- [27] M. Bycroft, K. J. Weissman, J. Staunton, P. F. Leadlay, *Eur. J. Biochem.* **2000**, *267*, 520–526.
- [28] D. A. Moffet, C. Khosla, D. E. Cane, *Bioorg. Med. Chem. Lett.* **2006**, *16*, 213–216.
- [29] B. Julien, Z.-Q. Tian, R. Reid, C. D. Reeves, *Chem. Biol.* **2006**, *13*, 1277–1286.
- [30] H. Liu, K. A. Reynolds, *J. Bacteriol.* **1999**, *181*, 6806–6813.
- [31] T. J. Erb, J. Rétey, G. Fuchs, B. E. Alber, *J. Biol. Chem.* **2008**, *283*, 32283–32293.
- [32] T. J. Erb, G. Fuchs, B. E. Alber, *Mol. Microbiol.* **2009**, *73*, 992–1008.
- [33] S. F. Haydock, A. N. Appleyard, T. Mironenko, J. Lester, N. Scott, P. F. Leadlay, *Microbiology* **2005**, *151*, 3161–3169.
- [34] R. Fouces, E. Mellado, B. Díez, J. L. Barredo, *Microbiology* **1999**, *145*, 855–868.
- [35] J. W. Vrijbloed, K. Zerbe-Burkhardt, A. Ratnatilleke, A. Grubelnik-Leiser, J. A. Robinson, *J. Bacteriol.* **1999**, *181*, 5600–5605.
- [36] Y. A. Chan, A. M. Podevels, B. M. Kevany, M. G. Thomas, *Nat. Prod. Rep.* **2009**, *26*, 90–114.
- [37] L. Diacovich, D. L. Mitchell, H. Pham, G. Gago, M. M. Melgar, C. Khosla, H. Gramajo, S.-C. Tsai, *Biochemistry* **2004**, *43*, 14027–14036.
- [38] C. Li, K. E. Roege, W. L. Kelly, *ChemBioChem* **2009**, *10*, 1064–1072.
- [39] Y. Xiao, S. Li, S. Niu, L. Ma, G. Zhang, H. Zhang, G. Zhang, J. Ju, C. Zhang, *J. Am. Chem. Soc.* **2011**, *133*, 1092–1105.
- [40] A. Arbolaza, M. E. Shillito, T.-W. Lin, L. Diacovich, M. Melgar, H. Pham, D. Amick, H. Gramajo, S.-C. Tsai, *Biochemistry* **2010**, *49*, 7367–7376.
- [41] Y. Kimura, T. Kojyo, I. Kimura, M. Sato, *Arch. Microbiol.* **1998**, *170*, 179–184.
- [42] V. V. Phelan, Y. Du, J. A. McLean, B. O. Bachmann, *Chem. Biol.* **2009**, *16*, 473–478.
- [43] L. M. Hicks, M. C. Moffitt, L. L. Beer, B. S. Moore, N. L. Kelleher, *ACS Chem. Biol.* **2006**, *1*, 93–102.
- [44] D. Meluzzi, W. H. Zheng, M. Hensler, V. Nizet, P. C. Dorrestein, *Bioorg. Med. Chem. Lett.* **2008**, *18*, 3107–3111.
- [45] K. Rinehart, M. Namikoshi, B. Choi, *J. Appl. Phycol.* **1994**, *6*, 159–176.
- [46] M. C. Moffitt, B. A. Neilan, *Appl. Environ. Microbiol.* **2004**, *70*, 6353–6362.
- [47] N. A. Magarvey, Z. Q. Beck, T. Golakoti, Y. Ding, U. Huber, T. K. Hemscheidt, D. Abelson, R. E. Moore, D. H. Sherman, *ACS Chem. Biol.* **2006**, *1*, 766–779.
- [48] L. Xiang, B. S. Moore, *J. Biol. Chem.* **2002**, *277*, 32505–32509.
- [49] J. S. Williams, M. Thomas, D. J. Clarke, *Microbiology* **2005**, *151*, 2543–2550.
- [50] M. C. Moffitt, G. V. Louie, M. E. Bowman, J. Pence, J. P. Noel, B. S. Moore, *Biochemistry* **2007**, *46*, 1004–1012.
- [51] T. Ahrendt, M. Miltenberger, I. Haneburger, F. Kirchner, M. Kronenwerth, A. O. Brachmann, H. Hilbi, H. B. Bode, *ChemBioChem* **2013**, *14*, 1415–1418.
- [52] P. M. Dewick, *Medicinal Natural Products: A Biosynthetic Approach*, Wiley, Chichester, **2009**, p. 116.
- [53] C. Hertweck, *Angew. Chem. Int. Ed.* **2009**, *48*, 4688–4716; *Angew. Chem.* **2009**, *121*, 4782–4811.
- [54] J. Sambrook, D. W. Russell, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York, **2000**.
- [55] A. Dereeper, V. Guignon, G. Blanc, S. Audic, S. Buffet, F. Chevenet, J.-F. Dufayard, S. Guindon, V. Lefort, M. Lescot, J.-M. Claverie, O. Gascuel, *Nucleic Acids Res.* **2008**, *36*, W465–W469.
- [56] A. Dereeper, S. Audic, J.-M. Claverie, G. Blanc, *BMC Evol. Biol.* **2010**, *10*, 8.

Received: October 23, 2013

Published online on February 13, 2014